Gap-Junctional Intercellular Communication in Epidermal Cell Lines from Selected Stages of SENCAR Mouse Skin Carcinogenesis

Richard C. Klann, D. James Fitzgerald, Colette Piccoli, Thomas J. Slaga, and Hiroshi Yamasaki


ABSTRACT

Homologous and heterologous gap-junctional intercellular communication (IC) was characterized in a panel of cell lines derived from selected stages of SENCAR mouse skin carcinogenesis. This panel included a "carcinogen-altered" cell line, 3PC, obtained from Ca²⁺-resistant primary adult keratinocytes after exposure to dimethylbenz(a)anthracene as well as cell lines obtained from early and late-stage papillomas and a squamous cell carcinoma (CA3/7) generated during standard in vivo initiation/promotion protocols (dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate). Also studied was a cell line (B66BA) obtained from a metastatic lesion following benzo(a)pyrene-induced skin tumorigenesis.

Intercellular communication was measured in low-calcium (0.05 mM) medium by quantitation of cell-cell transfer of microinjected fluorescent dye Lucifer Yellow CH. Homologous IC ability diminished progressively from 68 dye-coupled cells per injection for 3PC cultures, to between 21 and 54 dye-coupled cells per injection for three papilloma-derived cell lines, to six and three dye-coupled cells per injection for CA3/7 and B66BA cells, respectively. To test communication of these cells with their normal counterparts, heterologous IC was examined in cocultures with primary adult keratinocytes. Under the conditions used, normal cells established functional communication channels with each cell line tested, showing no selectivity. These results suggest that progressive loss of homologous but not heterologous IC capacity accompanies neoplastic development in mouse skin carcinogenesis.

INTRODUCTION

The direct cell-cell exchange of low-molecular-weight cellular components (MW, < 1000) that occurs via gap junctions is considered to play a pivotal role in the fundamental biological processes of development, tissue homeostasis, and differentiation (1–3). Not surprisingly, then, since cancer is essentially a derangement of these processes, the relationship between tumorigenesis and gap-junctional intercellular communication (IC) has been the subject of many studies. In early studies on tumor tissues, ionic coupling was measured to assay functional cell-cell communication, and gap junction structure was examined by electron microscopy. These studies revealed that many tumors are deficient in gap-junctional communication (4-6) or have fewer or qualitatively different gap junctions when compared with surrounding normal tissue (7, 8). These alterations are not seen in all tumors, however (7), just as tissue culture studies show that many but not all tumor cells are IC incompetent (2, 9–11).

Aside from diminished intrinsic IC competence of neoplastic cells, other modes of perturbation of IC include briefly: (a) defective responses of tumor cells to signals that modulate IC, as shown for mouse mammary tumor cells (12); (b) disturbed IC in normal tissue surrounding a tumor, as in the case of Novikoff hepatoma (4), implying an influence of some factor produced by the tumor on IC of normal cells; (c) altered specificity of IC of tumor cells, as shown by mammary cells (13), among others (14, 15), which acquire with transformation an ability to establish gap junctions with contacting fibroblasts both in vitro and in vivo, a change that may then allow exploitation of growth-stimulatory signals from this abnormal source; and (d) establishment of selective IC. Recent experiments showed that in situ foci formed by carcinogen- or oncogene-transformed BALB/c 3T3 cells display an internal IC capacity similar to that of surrounding normal cells but do not communicate with them (16–18). This selective IC could be abolished with such agents as retinoic acid and dibutyryl cyclic AMP and resulted in regression and disappearance of the transformed foci (19). Selective IC was also observed in coculture experiments with tumorogenic rat liver epithelial cells and nontumorogenic counterparts (20) and with pairs of normal/abnormal C3H110T½/⅔ cells (21). These studies suggested that the establishment of selective IC in an emerging neoplasm may aid the escape of tumor cells from controls on proliferation and differentiation normally imposed within a cell society. In addition to these findings of alterations of IC characteristics in neoplastic populations, the large body of data demonstrating inhibition of IC by many tumor promoters adds strength to the contention that disturbed IC may play an important role in carcinogenesis (22–25).

Although IC of normal and fully neoplastic populations has been compared in many cancer model systems, little information is available on IC changes during the progressive stages of neoplastic development (26, 27). Among the many model systems available, mouse skin presents an attractive choice for such studies because of its well-characterized stages of initiation and promotion (28). Recently, we established cell lines from papillomas and carcinomas of SENCAR mice after applying a treatment protocol of DMBA initiation and TPA promotion. We also used a cell line derived from a lymph node metastasis, as well as a "carcinogen-altered" cell line generated after exposure of adult mouse primary keratinocytes to DMBA and followed by selection in high Ca²⁺ medium. Acquisition of resistance to the differentiation-inducing effect of calcium has been suggested to be the critical event associated with initiation of carcinogenesis in mouse skin (29). In this report, we describe the IC characteristics of these cell lines derived during selected stages of carcinogenesis in mouse skin. We used fluorescent dye microinjection techniques to assay homologous IC in these cell lines and heterologous IC in cocultures established between them and primary adult keratinocytes.

Received 8/15/88; revised 10/27/88; accepted 11/2/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research was supported in part by Grant R01-CA-40534, National Cancer Institute, USPHS. Preliminary results of the work were presented at the 38th Annual Meeting of the Tissue Culture Association, Washington, DC, 1987 (see Ref. 56).

2 Supported by the Internal Research Data Bank Programme under Contract N01-CO-65341 (International Cancer Research Technology Transfer), National Cancer Institute, and the International Union Against Cancer. To whom requests for reprints should be addressed.

3 The abbreviations used are: IC, gap-junctional intercellular communication; DMBA, dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; FAV, fibronectin plus albumin plus vitrogen.

4 R. C. Klann, B. S. Beilue, C. L. Haskin, and T. J. Slaga. Establishment and characterization of cell lines from SENCAR mouse papillomas and carcinomas, submitted for publication.
MATERIALS AND METHODS

Cell Culture. The base medium used for primary and established cell lines was Eagle's minimal essential medium prepared from individual ingredients with the omission of CaCl₂. Components (Sigma Chemical Co., St. Louis, MO) added to this Ca²⁺-free medium were: insulin (5 μg/ml final concentration); epidermal growth factor (5 ng/ml); transferrin (10 μg/ml); O-phosphorylethanolamine (10 μM); 2-aminoethanol (10 μM); hydrocortisone (1 μM); gentamicin sulfate (50 μg/ml); and glucose (Gibco; 2 mM). This medium was then supplemented with 8% fetal bovine serum previously Chelex treated (Chelex-100 resin, Na⁺ form; BioRad Labs, Richmond, CA) to remove calcium (30). According to atomic absorption analysis, the Ca²⁺ concentration of this medium was approximately 0.03 mM. Calcium chloride was then added to give a final Ca²⁺ concentration of 0.05 mM, and this low-calcium medium was used for most experiments. When high-calcium medium was required, CaCl₂ was added to a final concentration of 1.2 mM.

Cell cultures were established in 60-mm dishes (Falcon) precoated just before use with FAV solution, essentially according to Kawamura et al. (31). To each dish were added 2.0 ml of a solution of bovine fibronectin (10 μg/ml; Sigma), bovine serum albumin (0.1 mg/ml; Sigma), collagen (30 μg/ml; Collagen Corp., Palo Alto, CA), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in Ca²⁺, Mg²⁺-free phosphate-buffered saline. The solution was then aspirated and the dishes left to air dry for 10 to 15 min before cell seeding. All incubations were conducted at 37°C in a humidified atmosphere of 5% CO₂.

Primary Cell Cultures. Cultures of primary keratinocytes were obtained from 6- to 9-week-old female SENCAR mice (Frederick Cancer Research Facility, Frederick, MD) as described previously (32). Typical cell yield and viability, determined by trypsin blue dye exclusion, were 15 x 10⁶ to 30 x 10⁶ cells/dorsal skin with viability of 60 to 80%. Viable cells (10 x 10⁶ to 30 x 10⁶) were seeded in 4 ml of medium onto FAV-coated dishes; after 24 h, cultures were refed with fresh medium and used for dye transfer studies 2 to 3 days later.

SENCAR Mouse Epidermal Cell Lines. The cell lines used in this study were considered to represent early, intermediate, and late stages of mouse skin carcinogenesis. The 3PC cell line was obtained after exposure of adult primary keratinocytes in vivo to DMBA (0.1 μg/ml, 24 h) and a shift to high-Ca²⁺ medium to select for Ca²⁺-resistant cells. Other investigators have similarly generated Ca²⁺-resistant carcinogen-altered keratinocytes following in vivo or in vitro carcinogen exposure (29, 32, 33). Papilloma-derived cell lines were obtained from tumors appearing after DMBA initiation of dorsal skin of 7- to 8-week-old female SENCAR mice followed by brief (P1/17) or extended (P2/15, P3/22) promotion with TPA according to the scheme depicted in Fig. 1. The CA3/7 cell line was obtained from a carcinoma 16 wk after completion of the DMBA/TPA regimen. Upon reintroduction in vivo in implantation chambers, the histological characteristics of the epithelia formed by these tumor cell lines reflected those of the original tumors; the papilloma-derived lines produced hyperplastic and hyperkeratotic epithelia, whereas CA3/7 cells produced highly invasive squamous cell carcinomas. The cell line B66BA was obtained from a lymph node metastasis sample after carcinoma formation induced by multiple benzo(a)pyrene (Bap) treatments.

Assay of Intercellular Communication. Gap-junctional intercellular communication was assayed using the method of Lucifer Yellow dye microinjection (34, 35). Injection needles were prepared from microfilament glass (A-M Systems, Everett, WA) using a dual-step puller (Narishige Co., Tokyo, Japan). Needles were then filled with a solution of 10% Lucifer Yellow CH (Sigma) in 0.33 M LiCl and fixed in the condensor of an inverted microscope (Olympus Injectoscope IMT-2 SYF). With the aid of an automatic (pneumatic) microinjector (Eppendorf, Model 5242), individual cells were injected with the fluorescent dye, and the extent of cell-cell spread of the dye was observed under fluorescence 15 to 20 min after injection. Communication in homologous cultures was tested 24 h after addition of fresh medium, and 15 to 30 independent injections were made for each experimental point. To study heterologous communication in cocultures, first- or second-order neighbors of a bead-labeled cell were injected, and after 10 to 15 min the labeled cell was scored as a dye recipient or not.

RESULTS

Homologous Communication Studies. In choosing the culture conditions for the present study, two important factors were culture substrate and medium calcium concentration. Clonal growth experiments indicated that the tumor-derived cell lines are not markedly sensitive to the type of substrate (plastic or FAV) or the calcium concentration (0.05 mM or 1.2 mM). Coculture experiments involving primary keratinocytes require more exacting conditions, however, because normal adult keratinocytes are difficult to culture on plastic or in the presence of high-Ca²⁺ medium (31, 36). In preliminary experiments, the effect of substrate and calcium concentration on IC was examined in three of the cell lines; these results are shown in Fig. 2. For 3PC, P1/17, and P2/15 in low-Ca²⁺ medium, IC was not significantly affected by substrate, although a slight increase was apparent for 3PC and P1/17 cultured on FAV-coated dishes. In addition, for 3PC, a shift to high-Ca²⁺ medium did not alter IC, regardless of the substrate. Since IC competence of these cell lines was generally unaffected by these culture parameters, we elected to use the conditions favoring primary cell culture, low-Ca²⁺ medium, and FAV substrate for further experiments.

The results shown in Fig. 2 suggested that the intrinsic IC capacity of the papilloma lines P1/17 and P2/15 was less than that of 3PC, the carcinogen-altered cell line. A comparative study of homologous IC involving all six cell lines is shown in Fig. 3. Evident is a progressive decline in IC competence that correlated with the successive stages of skin carcinogenesis (see also Fig. 4). Early stage 3PC cells communicated best, transfer-
ring dye to an average of 68 neighboring cells. Dye transfer in cultures of papilloma-derived cells nearly matched this level in early stage P1/17 papilloma cells (54 recipients per injection), whereas cells from progressively later stages showed a decrease from 28 dye-coupled cells per injection in the P2/15 cultures to 22 recipients for the P3/22 cell line. Cell lines representing the last stages of the process of carcinogenesis, squamous cell carcinoma (CA3/7) and metastasis (B66BA), showed marked reduction of communication to six and three recipients per injection, respectively. When assayed with this methodology, primary cultures of normal mouse keratinocytes demonstrated an intermediate IC capacity, 15 to 30 recipients per injection. Directly comparing this to the IC of the cell lines may be inappropriate, however, because, in addition to the obvious difference in their adaptation to culture conditions, the primary cultures also comprise a heterogeneous population of cells.

We further characterized the homologous IC of cell lines 3PC and CA3/7 by assaying communication during the growth of cultures to confluence. As shown in Fig. 5, IC was differentially modulated by degree of confluence in these two cell lines. At subconfluence, CA3/7 cells were communication competent, but this property was progressively lost as the cells approached the confluent state. In 3PC cells, conversely, IC capacity was initially relatively low but increased with time.

Heterologous Communication Studies. We next examined the cell lines' ability to communicate with normal keratinocytes. Considering that preneoplastic and neoplastic cells coexist with normal cells in vivo, we sought to establish the IC relationship between cancerous or potentially cancerous cells and surrounding normal cells in vitro. In these coculture experiments it was necessary to tag one of the cell types because normal keratinocytes and cell lines in culture cannot be distinguished by definitive morphological characteristics. Tagging was done as described in “Materials and Methods.” Labeled and dispersed cell lines were added to primary cell cultures and allowed various coculture intervals. Primary cells located near tagged cells were then microinjected with Lucifer Yellow, and the latter were noted as having received the dye or not. As a control, labeled cells were replated alone, and their ability to establish homologous junctional contacts was assessed after the same incubation periods used for the cocultures. In all controls, homologous communication was evident. As shown in Fig. 6 and Table 1, these communication-competent labeled cells also displayed heterologous communication with the surrounding lawn of normal cells in the cocultures. Dye transfer was observed at 85 to 100% of the heterologous sites (Table 1).

To see whether the same was true for heterologous populations containing an excess of cell-line cells rather than normal cells, cocultures were prepared with labeled primary keratinocytes added to near-confluent cultures of 3PC or CA3/7 cells. The results shown in Table 2 indicated that, upon Lucifer Yellow introduction into transformed cells, nearby normal cells subsequently received the dye. Thus, under these experimental conditions, the establishment of heterologous gap junctions was evident regardless of the ratio of the cell types in coculture or the nature of the cell type receiving the dye injection. In similar studies, we also observed heterologous communication in cocultures of normal and transformed human keratinocytes.5

DISCUSSION

The principal findings in the present comparative study of homologous IC in these mouse epidermal cell lines suggested that communication capacity decreases in a progressive manner during skin carcinogenesis. The communication assay used, however, measured only the number of dye-coupled cells after each microinjection. Since the total area of dye transfer was not quantitated, the same result could also be obtained if there were major differences in cell size at confluence among the cell lines. In a comparison of two cell populations of widely different cell size and a similar area of dye transfer, the smaller-celled population would seem to be the more communicative. This is unlikely to be true of our results, however, since no major differences in cell size were observed. Moreover, visual assessment of dye-transfer area showed the same differences among the cell lines as we noted using the cell number method (see Fig. 4). Future studies using computerized image analysis in determining the rates of cell-cell dye transfer should clarify ambiguities (37–39).

The implications of the observed pattern of decreased IC in cells from later stages of carcinogenesis are currently not clear. Presumably, as a neoplasm progresses, its constituent cells express greater independence and irreconcilability (23, 40). Insofar as gap-junctional communication is concerned, reduced cross-talk between neoplastic and normal cells could weaken...
the normalizing influences emanating from the surrounding tissue. Such normalizing effects have been observed in various *in vitro* and *in vivo* studies of normal cells placed in contact with premalignant or malignant cells (41-48). A disruption of these influences could occur *in situ* if heterologous communication between tumor and host cells were abolished, or if homologous communication among the cells of an emerging neoplasm were reduced. Our current data are consistent with the latter hypothesis; however, whether progressive reduction of IC, as seen in the cell lines studied, represents a cause or effect of epidermal carcinogenesis cannot be determined from the results of this study. Decreased homologous IC capacity was recently shown to correlate with the degree of transformation in rat liver epithelial cell lines (10) and the degree of metastatic potential of rat mammary adenocarcinoma cell lines (49).

The measurements of homologous IC were conducted in confluent cultures. Although it is not known which *in vitro* situation best simulates intact skin, we assume that, in terms of cell-cell contact, confluence *in vitro* is a fair representation. Nonetheless, homologous IC of 3PC and CA3/7 cells was also tested at subconfluence (Fig. 5). Under these conditions the
carcinoma-derived cells were communication competent but rapidly lost IC ability as confluence was reached. In striking contrast, 3PC cultures displayed increased IC with increased cell density. This finding clearly indicated that these cell lines differ greatly in the regulation of communication. Although these differences cannot be explained on the basis of the findings presented, an association with carcinogenesis is possible. The transition from good to poor communication in CA3/7 cells may involve a fundamental pathway of IC regulation, for example, increased synthesis of some factor, knowledge of which could help to uncover new methods of cancer prevention. Preliminary experiments have suggested that CA3/7 cells cocultured with a large excess of 3PC cells can decrease the IC capacity of the latter. Whether this decrease is mediated by an agent that is secreted, transmitted through gap junctions, or is acting through some other mechanism is not yet known. Such a phenomenon was described by Loewenstein and Kanno (4), who found reduced IC capacity in normal cells surrounding induced or transplanted liver tumors.

Our experiments suggested progressive reduction of homologous IC capacity during skin carcinogenesis, but heterologous IC with normal keratinocytes was maintained in all cell lines. Theoretically, to explain the suppression of clonal expansion of initiated cells in situ (23, 24), 3PC, the carcinogen-altered cell line, would be expected to communicate with normal cells. For the tumor-derived cell lines, selective communication may be unnecessary because reduced homologous IC may weaken normal cell influences sufficiently to allow expression of the transformed phenotype. Recent studies with C3H10T1/2 (22) or BALB/c 3T3 fibroblasts (19, 46) demonstrated, however, that these transformed cells avoid growth suppression by selective communication that excludes their nontransformed neighbors. These results, however, may not accurately reflect the pattern of IC occurring during carcinogenesis when analyzed using primary cultures of normal and tumor tissues, because of the very abnormal nature of these heteroploid cell lines.

Alternatively, this disparity may reflect a fundamental difference between carcinogenesis in epithelial and mesenchymal cell systems. Transformed foci of BALB/c 3T3 cells display no
This could be so if some stricture of the gap-junction channels had occurred, perhaps as a result of defective alignment of heterologous junction moieties. Probes of different sizes (up to \( M_r 1,000 \)) could be used to test this (51).

The system we used in these studies offers an excellent opportunity to identify changes associated with tumor development, since neoplastic and preneoplastic cells from defined stages of initiation and promotion can be manipulated for analysis. Recently, much work has been done to characterize genetic changes related to mouse skin carcinogenesis, particularly the activation of the H-ras oncogenes by DMBA (52–55). We are currently exploring how these alterations relate to gap-junctional communication changes. In addition, it will be important to determine the homologous and heterologous cell-cell communication characteristics of emerging tumors in vivo, and such studies are in progress.

ACKNOWLEDGMENTS

We thank Aurora Viaje for provision of the 3PC cell line; M. Imbert, Service Central de Microanalyse, Solaize, France, for atomic absorption determinations of medium calcium concentrations; and C. Fuchez for preparing the manuscript.

NOTE ADDED IN PROOF

Further evidence for reduced IC in the late stages of skin carcinogenesis comes from recent studies in our laboratory using three additional carcinoma-derived cell lines, CA 4/5, CA 8/29 and CA 9/20. These cells demonstrate homologous IC of 3.7 ± 0.3, 13.2 ± 0.9 and 2.8 ± 0.4 recipients per injection, respectively.

REFERENCES


Gap-Junctional Intercellular Communication in Epidermal Cell Lines from Selected Stages of SENCAR Mouse Skin Carcinogenesis


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/49/3/699

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/49/3/699. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.